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(54) Title: METHODS AND COMPOSITIONS FOR GENOTYPING CATTLE FOR RED FACTOR

(57) Abstract

Provided are methods, and compositions for carrying out the methods, for determining whether a gene for the red factor, which provides red coat color, is present in the genome of a domesticated mammal, especially one of genus *Bos* (cattle). The methods can be applied with DNA obtained from gametes, zygotes, cells, embryos, fetuses or whole animals. The methods are based on the discovery that coat color genes in genus *Bos* are alleles of the gene for the melanocyte-stimulating hormone receptor and the discovery of primers that permit amplification of the segment of that gene that includes the differences between alleles that provided black hair color and those that provide red hair color.

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METHODS AND COMPOSITIONS FOR GENOTYPING CATTLE FOR RED FACTOR

TECHNICAL FIELD

The present invention concerns coat color in mammals, especially cattle, domesticated mammals of genus *Bos*.

More particularly, the invention concerns methods and compositions for determining whether a gamete, zygote, cell, embryo, fetus, or whole animal, especially of genus *Bos*, has in its genome a gene for the red factor.

BACKGROUND OF THE INVENTION

Red coat color in domesticated mammals, including cattle (animals of genus Bos), is a recessive trait. The corresponding dominant trait is black coat color. An animal which is homozygous for alleles of the gene, which determine coat color, that provide red hair, and therefore red coat color, will have a red coat. An animal which is heterzygous for such alleles, and consequently has an allele that provides black hair, and consequently black coat color, on one of the pair of relevant chromosomes, and an allele that provides red hair on the other of the pair of chromosomes, will have a black coat but will be a red factor carrier capable of passing a red factor allele to its progeny. Finally, an animal which is homozygous for alleles which provide black hair also will have a black coat.

Heretofore it has not been possible to determine the genotype for coat color of the genome of a domesticated mammal, including cattle. This is both because the gene, of which various possible alleles determine coat color, had not been determined for domesticated mammals, including cattle, and because no method for distinguishing between alleles of that gene

that provide red hair and alleles of that gene that provide black hair was known.

Nonetheless there has been considerable interest in the breeding industries for domesticated mammals, including cattle, to have a test to determine the genotype for coat color of genomes in various situations. For example, among cattle, animals carrying an allele for red coat color command an higher price. Therefore there has been interest in the cattle breeding industry to determine whether a particular bull or cow is capable of providing calves with red coat color and whether an embryo will develop into an individual with a red coat color.

Thus, it has been recognized that, if it were possible, testing of semen for sperm which harbor a red or black factor allele and testing of cells, such as leukocytes, from animals for genomes that have a red factor allele would be of considerable value.

It has been recognized that the ability to test the genotype of cells from embryos at or before the stage (about 32 cells) at which all of the cells remain totipotent would be valuable as well. In these stages (32 or fewer cells), embryos may be split to provide both cells for genotyping and cells for development through gestation into whole animals.

SUMMARY OF THE INVENTION

We have now discovered that alleles of the melanocyte-stimulating hormone receptor gene in cattle determine the coat color of the cattle.

Numerous breeds of cattle have been examined, and in all of them the coat color is due to alleles of the melanocyte-stimulating hormone receptor gene.

Furthermore, we have discovered that the sequence differences among the alleles occur in a segment of about 600 base pairs of the melanocyte-stimulating hormone receptor gene and that this segment can be readily amplified by any of various amplification protocols now available in the art (including PCR) using as primers

those that comprise SEQ ID NO:2 (or any subsegment of 12 or more bases thereof) (for forward priming) and those that comprise SEQ ID NO:1 (or any subsegment of 12 or more bases thereof) (for reverse priming).

Finally, we have discovered that single-stranded DNAs (or RNAs) from the amplifications of the alleles for red factor can be readily distinguished from the single-stranded DNAs (or RNAs) from amplifications of the alleles for black factor because of conformational differences among these single-stranded nucleic acids.

More particularly, the invention entails a method for determining the coat-color genotype of a genome of genus *Bos* (in a gamete (haploid genome), zygote, cell, embryo or embryonic cell, fetus, or cells from a whole animal (blood, skin, etc.) which comprises determining the coat-color-determining alleles of the melanocyte-stimulating hormone receptor genes of the genome.

Typically, the method will entail amplifying the segment of said gene which includes the allelic differences among the coat-color-determing alleles by any of the various amplification methods available in the art (including PCR) and then analyzing the amplified nucleic acid (which may be RNA or, particularlty if PCR amplification is used, DNA) for which allele is present.

The invention also entails primers, individually and in combination, that may be emplyed to carry out the amplification methods useful in the invention. Thus, among others, the invention entails a DNA with a sequence selected from the group consisting of the sequence of SEQ ID NO:1, or any subsegment of at least 12 bases thereof, and the sequence of SEQ ID NO:2, or any subsegment of at least 12 bases thereof. Further, the invention entails a composition which is an aqueous solution of any pair of such primers which is capable of amplifying a segment of the melanocyte-stimulating hormone receptor gene in the genome of a cattle species.

EXAMPLE

DNA is isolated by any standard procedure from embryonic cells, blood cells (leukocytes, sperm or the like.

DNA used in this analysis can be obtained using standard procedures from, but not limited to, whole blood, leukocytes (isolated from whole blood or milk), semen, skin, organ tissue, embryonic stem cells, embryos, aborted fetuses and amniotic fluid. Standard methods exist for isolating DNA from all of these sources are easily performed in a standard molecular genetics laboratory. Once DNA has been isolated, the purity is checked spectrophotometrically by comparing the absorbance at 260 nm to the 280 nm absorbance. A 260 nm/280 nm ratio of 1.6 to 2.0 is indication of sufficient purity to perform the analysis. The absorbance at 260 nm is used to determine the DNA concentration using the conversion factor 50μg = 1 A_{260}.

The analysis method preferably used entails amplification by the polymerase chain reaction (PCR). The reactions can be performed in either 0.5 ml microcentrifuge tubes, tube strips, 96-well microtiter plates or other conventional formats that are readily incubated in commercially available thermocyclers (i.e., MJ Research, Hybaid Omnigene, Perkin Elmer, etc.). A standard single reaction contains 50 - 200 nm of DNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.5 μM of each oligonucleotide primer (SEQ ID NO:1 and SEQ ID NO:2) and 0.5 U Taq polymerase in a final volume of 25 μl. For optimal results, the reaction is performed using a "hot start" procedures, where the Tag polymerase is added to each reaction during the first denaturing step. The cycling parameters are 3 min at 98°C (the first denaturing step), followed by 2 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 73°C, followed by 4 cycles of 1 min at 94°C, 1 min at 59°C and 1 min at 73°C, followed

by 4 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 73°C, followed by 4 cycles of 1 min at 94°C, 1 min at 55°C and 1 min, 30 sec. at 73°, followed by 15 cycles of 1 min at 94°C, 1 min at 56°C and 1 min, 30 sec at 73°C, and ending with a 5 min extension step at 73°C. In order to optimize the procedure, slight modifications of the above procedure may be necessary depending on the amount and/or source of DNA available, the type of incubation format used, the source of Taq polymerase and/or the type of thermocycler used, as well understood in the art.

Analysis of the PCR product can be performed using many methods. Initially, the reaction products are analyzed on a 1.5% agarose, 1 X TBE (Tris, borate, EDTA) gel to confirm amplification of the appropriate size DNA product. Ethidium bromide staining of the agarose gel should reveal a single fragment approximately 600 base pair long. After confirming amplification, a 5 μl sample of the reaction product is mixed with a 5 μl of stop solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) and 1 μl of 10 mM EDTA pH 8.0, 0.1% SDS, incubated for no less than 10 min at 95°C and placed immediately on ice. This chilled mixture is loaded on a 10% polyacrylamide gel (37.5:1 acrylamide:bis-acrylamide) and electrophoresed 16-24 hr. at 15-20 Volts/cm.

Visualization of the alleles is accomplished by several methods. If the oligonucleotide primers are labeled at the 5' end with ^{32}P, or if radiolabeled $\alpha^{32}P$-dATP is included in the reaction mixture, the gel can be dried and exposed to autoradiography film to reveal the alleles. Alternatively, the gel can be stained with either ethidium bromide or a fluorescent dye, such as SYBR Green (Molecular Probes), and then visualized by UV illumination at 254 nm and photographed using a red filter (ethidium bromide) or green filter (for SYBR Green). Gels stained with ethidium bromide or SYBR Green can also be visualized by fluorescence emission using fluorescent based detectors such as the Molecular

Dynamics Fluorimager (Figure XX). The alleles can also be visualized by silver staining methodology using either commercially available silver staining kits (BioRad) or "home made" silver staining protocols and reagents.

The separation methods presented here are not exhaustive. Other gel matrices are available to separate the alleles, such as specialized high resolution agarose (i.e., MetaPhor XR, FMC BioProducts) and specialized acrylamide derivatives (i.e., Mutation Detection Enhancement Gel, J.T. Baker), and could be used once optimized. Other visualization techniques are also available, such as fluorescence labeling of oligonucleotide primers combined with fluorescence emission detection, biotinylation of oligonucleotide primers in conjunction with secondary avidin or stepavidin enzyme (alkaline phosphatase, horse radish peroxidase, etc.) conjugate binding and colorimetric detection of enzymatic products, hapten-primary antibody and secondary antibody-conjugate binding used in colormetric assays or Southern blotting methods were radiolabeled probes are used to detect DNA alleles bound to nylon or nitrocellulose membranes.

Single-stranded DNA segements from the amplification migrate at different rates in the gel, presumably because of conformational differences arising from sequence differences, for the different alleles. Thus, the alleles are subject to the type of analysis described here, a form of SSCP analysis as well known in the art.

Three bands have been detected in conjunction with allelic variation of the melanocyte-stimulating hormone gene in determining coat color. The slowest moving band (Band 1) is a non-specific isolate that is common to all samples tested, thus is not indicative of genotype or phenotype. Band 2 segr gates with either the red homozygous or red carrier (heterozygous) conditions. Band 3 segregates with either the black homozygous, black

heterozygous or red carrier conditions. Thus the genotypes are determined as follows:

Band 3 only	Homozygous Black
Band 2 and 3	Heterozygous Red (Red Carrier, RC)
Band 2 only	Homozygous Red

Of course, the location of the various bands is determined by running appropriate controls from homozygous black, homozygous tred, and red carrier uindividuals.

SEQUENCE LISTING

SEQ ID NO:1

5'- AAGAGGTTGA AGTTCTTGAA GATGC (reverse primer)

SEQ ID NO:2

5'- CATGTACTGC TTCATCTGCT GCC (forward primer)

CLAIMS

1. A method for determining the coat-color genotype of a genome of genus *Bos* which comprises determining the coat-color-determining alleles of the melanocyte-stimulating hormone receptor genes of the genome.

2. A method of Claim 1 which comprises amplifying the segment, which comprises the coat-color-determining allelic differences, of the DNA of the melanocyte-stimulating hormone receptor genes and analyzing the nucleic acid products of the amplification to determine the coat-color-determining alleles of those genes.

3. A method of Claim 2 wherein the amplification is by polymerase chain reaction.

4. A method of any one of Claims 1 - 3 wherein the genome is that of cells of an embryo.

5. A method of Claim 4 wherein the genome is that of totipotent cells of an embryo.

6. A method of any one of Claims 1 - 3 wherein the genome is that of somatic cells of a whole animal.

7. A method of Claim 5 wherein the somatic cells comprise cells from whole blood.

8. A method of Claim 5 wherein the somatic cells comprise cells from milk.

9. A method of Claim 5 wherein the somatic cells comprise skin cells.

10. A method of Claim 3 wherein the primers employed in the amplification comprise the primer of SEQ ID NO:1 and the primer of SEQ ID NO:2.

11. A method of Claim 4 wherein the primers employed in the amplification comprise the primer of SEQ ID NO:1 and the primer of SEQ ID NO:2.

12. A method of Claim 5 wherein the primers employed in the amplification comprise the primer of SEQ ID NO:1 and the primer of SEQ ID NO:2.

13. A method of Claim 6 wherein the primers employed in the amplification comprise the primer of SEQ ID NO:1 and the primer of SEQ ID NO:2.

14. A method of Claim 7 wherein the primers employed in the amplification comprise the primer of SEQ ID NO:1 and the primer of SEQ ID NO:2.

15. A method of Claim 8 wherein the primers employed in the amplification comprise the primer of SEQ ID NO:1 and the primer of SEQ ID NO:2.

16. A method of Claim 9 wherein the primers employed in the amplification comprise the primer of SEQ ID NO:1 and the primer of SEQ ID NO:2.

17. A method of Claim 2 or Claim 3 wherein the analysis of nucleic acid products is by SSCP.

18. A method of Claim 4 wherein the analysis of nucleic acid products is by SSCP.

19. A method of Claim 5 wherein the analysis of nucleic acid products is by SSCP.

20. A method of Claim 6 wherein the analysis of nucleic acid products is by SSCP.

21. A method of Claim 7 wherein the analysis of nucleic acid products is by SSCP.

22. A method of Claim 8 wherein the analysis of nucleic acid products is by SSCP.

23. A method of Claim 9 wherein the analysis of nucleic acid products is by SSCP.

24. A method of Claim 10 wherein the analysis of nucleic acid products is by SSCP.

25. A method of Claim 11 wherein the analysis of nucleic acid products is by SSCP.

26. A method of Claim 12 wherein the analysis of nucleic acid products is by SSCP.

27. A method of Claim 13 wherein the analysis of nucleic acid products is by SSCP.

28. A method of Claim 14 wherein the analysis of nucleic acid products is by SSCP.

29. A method of Claim 15 wherein the analysis of nucleic acid products is by SSCP.

30. A method of Claim 16 wherein the analysis of nucleic acid products is by SSCP.

31. A DNA with a sequence selected from the group consisting of the sequence of SEQ ID NO:1 and the sequence of SEQ ID NO:2.

32. In combination in an aqueous solution, a DNA with the sequence of SEQ ID NO:1 and a DNA with the sequence of SEQ ID NO:2.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05622

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C07H 21/04

US CL : 435/6; 536/24.3, 24.31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/24.3, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Proceedings of the National Academy of Sciences, USA, Volume 86, issued April 1989, M. Orita et al., "Detection of Polymorphisms of Human DNA by Gel Electrophoresis as Single-Strand Conformation Polymorphisms", pages 2766-2770.	1-30
X Y	Cell, Volume 72, Number 6, issued 26 March 1993, L. S. Robbins et al., "Pigmentation Phenotypes of Variant Extension Locus Alleles Result from Point Mutations that Alter MSH Receptor Function", pages 827-834, see pages 827, 831-832.	1-9 10-30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Genetic Analysis: Techniques and Applications, Volume 11, Number 1, issued 1994, G. C. Russell, " Improved Single-Strand Conformation Polymorphism Analysis by Asymmetric Polymerase Chain Reaction with End-Labeled Primers", pages 24-27, see entire document.	1-30
Y X	FEBS Letters, Volume 348, Number 3, issued 1994, M. Vanetti et al., "Molecular Cloning of a Bovine MSH Receptor which is Highly Expressed in the Testis", pages 268-272, see entire document, especially Figure 1.	1-30 31-32
Y X	Science, Volume 257, issued 28 August 1992, K. G. Mountjoy et al., "The Cloning of a Family of Genes that Encode the Melanocortin Receptors", pages 1248-1251, see pages 1248-1249, especially Figure 2A. Note, pertinant portion of GenBank/EMBL nucleotide sequence submission referred to in Fig. 2A has been attached.	1-30 31-32
Y	Animal Genetics, Volume 24, Number 2, issued 1993, G. L. Hart et al., "Detection of a four-allele single strand conformation polymorphism (SSCP) in the bovine prolactin gene 5' flank", page 149, see entire document.	1-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05622

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, CABA, CAPLUS, MEDLINE, EMBL, GenBank
search terms: Bos, taurus, cattle, cow#, steer, bovine, bovid, MSH-R, MSH receptor, melanocyte, melanocyte stimulating hormone receptor, melanocortin, melanocortin receptor, SSCP, mouse, coat, pelt, pelage, hair, fur